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Deuterium depleted water effect on *Euphorbia canariensis* L. micropropagation

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SUMMARY. It has been studied the effect of deuterium depleted water (DDW) (with 25 ppm D) on the culture of *Euphorbia canariensis* L., to transfer *ex vitro*. Distilled water (DW) (with 155 ppm D) from the culture medium of *Euphorbia* was replaced with DDW. Hypothesis of the present experiment was that presences of DDW in culture medium inhibit callus formation, stimulates rhizogenesis and optimizes *ex vitro* acclimatization of *in vitro* cultivated plantlets. After 18 months of *in vitro* culture - when the plantlets were transferred into soil - only those grown on medium prepared with distilled water showed an intense callusogenesis process at the level of the areolas and at the apical and basal poles. DDW stopped callus formation at this species and permitted rhizogenesis. Also, the chlorophylls *a*, *b* and carotenoids extracted in N, N-dimethylformamide - were increased in the presence of DDW in culture medium. At the end of the acclimatization, *ex vitro* survival rate of the plantlets from medium prepared with DDW, increased at 92%. *In vitro* plantlets cultivated on DW containing medium could not be transferred into a septic medium of life due to the lack of a radicular system.

Keywords: acclimatization, chlorophylls, deuterium, *Euphorbia canariensis*, micropropagation

Introduction

Euphorbiaceae species are an important source of dyes, oil, furniture, and rubber. From their latex extract are obtained diterpenoids used in pharmaceutical and chemical products that are useful for regulating blood vessel walls. *Euphorbia* is a genus of ecological, aesthetic and economic importance, due to its role in obtaining fuel and also as parent stock to other more sensitive species of the *Euphorbiaceae* family (Marco *et al.,* 1997; Jakupovic *et al*., 1998; Gaal *et al*., 2013).

In vitro multiplication and conservation techniques have economical and ecological importance. This type of cultivation and conservation is fast, efficient, modern and widely used. Germplasm cryopreservation is important for maintaining

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the genetic diversity of rare plants. Improved cell and tissue culture technologies would help producing the *in vitro* active compounds with better productivity, without decreasing natural resources (Kondamudi *et al*., 2009).

There are some opinions in the field of plant tissue culture suggesting that multiplication of *Euphorbiaceae* can and should be more developed (Balotis and Papafotiou, 2003; Toress, 2004; Bidarigh and Azarpour, 2013).

DDW has 25 ppm deuterium, comparatively with DW which has approximately 155 ppm D. Pacific Ocean water or Antarctic Region water has a lower deuterium contend (89 ppm) (Ignatov and Mosin, 2013). The authors studied the particularities of deuterium and the conditions of primary hydrosphere and tried to identify the origin of life and living matter.

Deuterium depleted water began to be used extensively for its effects to inhibit mitotic division of animal cancer cells, but it was also used to observe the influence on different plant species cultured *in vitro*. Deuterium depleted water (DDW) was known from our previous researches for having, in some cases, the effect of timing the growth (Cachiță *et al*., 2002; Butnaru *et al.*, 2004; Petruș–Vancea, 2011 a; Petruș-Vancea *et al*., 2013).

The chloroplast ultrastructure and assimilating pigments content at sugar beet *in vitro* formed leaflets was normal in the presence of deuterium depleted water in culture medium, because this treatment prevented hyperhydricity (Cachiță *et al.,* 2009).

At *Robinia pseudoacacia* var. Oltenica, deuterium depleted water rejuvenated the plant tissues by inhibiting basal callus formation, improving micropropagation, the method providing biological material in case of global climate change (Corneanu *et al.*, 2010).

In vitro studies on this species of *E. canariensis* are few (Tripon *et al.,* 2015), which is why we intend to realize this experiment. In the present study we propozed to identify the effect of deuterium depleted water on *E. canariensis* inoculs, to improve rhizogenesis process by stopping callusogenesis but also by stimulating the assimilating pigments synthesis for optimizing their adaptation to the septic medium of life after the *ex vitro* transfer.

Materials and methods

Initial *plant material* consisted in *Euphorbia canariensis* seeds germinated on standard basal medium Murashige-Skoog (1962) with Gamborg *et al*. (1968) ½ vitamins, solidified with agar-agar, without growth regulators and aminoacids, with 20 g/l sucrose (Tripon *et al*., 2015) instead of 30 g/l as in the original recipe.

Replication – using 0.5 – 0.7 cm inocula, respectively 3 nods – was made at 120 days from *in vitro* initiation (Fig. 1). The cuttings were placed on medium prepared with distilled water (control) (V_0) or with deuterium depleted water (DDW) (V_1) (Table 1).

Research design is presented in table 1.

After inoculation, the culture was incubating at 22-23 ˚C temperature, lighting with 1700 lx and 16/24h photoperiod.

Assimilating pigments measurement. Assimilating pigments measurement, respectively chlorophylls (Chl *a*, *b*) and carotenoids (Car), was made by extracting them from plantlet stem in N, N-dimethylformamide (DMF) 99.9%, according to the method developed by Moran and Porath (1980). Assimilating pigments quantitative values were obtained by using the absorption coefficients (Wellburn, 1994), measured at 664 nm for determination of Chl *a*, at 647 nm for Chl *b* content and 480 nm for carotenoids. Spekol 11 type, Carl Zeiss Jena spectrophotometer were used.

Table 1.

Experimental protocol concerning multiplication, conservation and acclimatization of *E. canariensis* (DW – distilled water; DDW – deuterium depleted water; G –Gamborg vitamins; MS –Murashige-Skoog basal medium).

Experimental steps	**Plant material**	**Culture Vessels**	**Culture medium**	**Culture and measurement period**	**Measured**
Step I *in vitro* initiation (first culture)	Seeds	7/2 cm size	MS ½ – G ½- DW	4 months	stem length nods number
Step II *in vitro* conservation	cuttings	7/2 cm size	V_0: MS – G – DW V_1: MS – G – DDW	18 months	roots length roots number total plantlets length (stem + roots) chlorophylls carotenoids
Step III *ex vitro* acclimatization	Plantlets with roots (from V_1)	pots placed in incubator	nutritive soil : sand 1:1	1 month	survival rate

Ex vitro acclimatization of *E. canariensis* was done at 18 months from *in vitro* culture subcultivation. Vitroplants were placed in individual containers. Since only vitroplants subcultured on media with deuterium depleted water (V_1) developed a vigorous root system, only they were transferred in the septic medium. Vitroplantlets without roots or a poorly developed root system, as those of control group (V_0), had no chance of survival in the *ex vitro* acclimatization. Lack of roots of these vitroplantlets was caused by the presence of callus at the base of inoculs. *Ex vitro* acclimatization conditions were identical to those held in *in vitro* incubating period cultures.

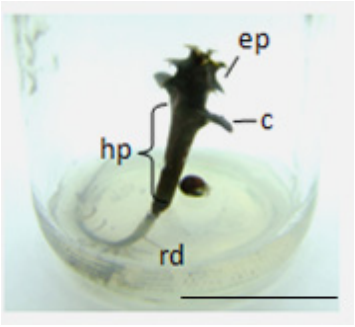

Figure 1. Aspect of *E. canariensis* seed plantlet, to 120 days from initiation (bar mean 1cm) (c – cotyledon; ep – epicotyl; hp – hypocotyl; rd – radicle).

Measurement results were mathematically (average, standard deviation and dispersion) and statistically (*t* test) processed using SPSS 16.0 for Windows Program. For each experimental variant three measurements were performed.

Results and discussions

At 18 months of in vitro culture, the total plantlets length did not have high growth (Table 2). At samples where distilled water was replaced with deuterium-depleted water (V_1) the rhizogenesis was intense.

Instead, the stem length was inhibited by the presence of deuterium depleted water in the culture medium (Table 2), as in the case of *Asparagus* species and *Beta vulgaris, Drosera rotundifolia, Cymbidium hybridum* (Petruş-Vancea, 2011 a, b).

Table 2.

E. canariensis plantlets statistical data processing at 18 months after inoculation on different culture media, namely: V_0 – MS culture medium prepared with distilled water (DW); V_1 - MS culture medium prepared with deuterium depleted water (DDW).

V_0-DW (control)				
Statistic data	**Roots length**	**Roots number**	**Stem length**	**Plantlets size**
X ± Sx	0.21±0.25	0.42 ±0.51	2.14±0.31	2.35±0.40
S^2	0.06	0.26	0.10	0.16
V_1-DDW				
X ± Sx	1.58±0.51	2.05±0.52	1.55±0.37	3.13±0.62
±d	+0.79	+1.16	-0.64	+0.15
S^2	0.26	0.27	0.14	0.39
P	**	**	*	**

Note: $\overline{x} \pm S_{\overline{x}}$ [average (cm) ± standard deviation], ±d [difference against control (cm)], s^2 – dispersion, p – significance: * - significantly ($p < 0.05$), ** - very significantly ($p < 0.01$).

In the present study, at 18 months of subcultures, only the plantlets grown on distilled water containing medium showed an intense process of callusogenesis at the level of the areolas. Callusogenesis process affected 27% of control lot inoculs (V_0). The deuterium depleted water (25 ppm D) stopped callus formation at this species and permitted rhizogenesis (Fig. 2 b and c), as in the case of sugar beet (Cachiță *et al.*, 2008; Petruş - Vancea and Cachiță, 2011).

Stopping callus formation is induced by DDW, because this type of water inhibits cell division, which is why it is used in the treatment of human cancer.

Callus lack at basal level of inoculum favored the formation of *in vitro* roots. A well-developed root system subsequently optimized adaptation of plantlets in septic medium, in *ex vitro* acclimatization stage.

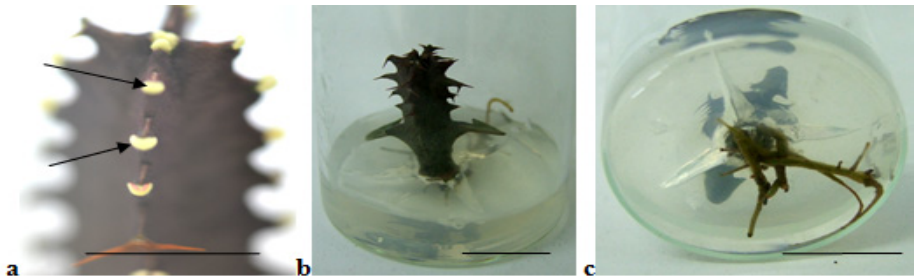

Figure 2. Growth of *E. canariensis* plantlets at 18 month on medium MS-G, prepared with distilled water (V_0 - control) (a) (arrows indicate callus formation at areola level), respectively on MS-G medium prepared with deuterium depleted water (V_1) (b and c) (bars indicate 1 cm).

Although some plantlets showed a brown skin, others were green as you can find in figure 3 (a and b), in the cross section we have seen that both groups had viable tissues (Fordon and Petruş-Vancea, 2015), even if the callus was stopped due to increased stem length. At other family, namely *Cactaceae*, this phenomenon also occurs under natural conditions of life, the appearance of necrotic stem doesn't show its lack of viability. Basically it is an adaptation to a harsh environment in which they live.

Assimilating pigments from stem, measured at 18 month of culture, showed that there was no significant difference between plantlets growth on DDW medium compared with those cultivated on medium prepared with DW, except carotenoids with a notable increase (74% percent) of its quantitative value at the lot containing DDW (Table 3). Also, the difference between the ratios Chl *a*/*b* was statistically insignificant at both lots of plants tested.

At 30 days of a*cclimatization*, post-acclimatization survival percent was calculated and the result was 92% (Fig. 4), where 100% represents the total number of vitroplantlets transferred into soil.

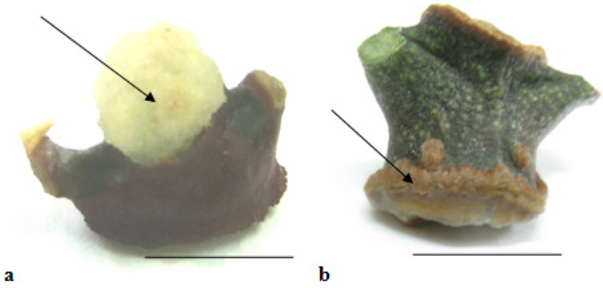

Figure 3. Callus formation (indicate by arrows) at apical level (a) and basal level (b) of *E. canariensis* inoculs, at 18 months of culture on MS-G medium prepared with distilled water (V_0 - control) (bars means 1 cm).

Table 3.

Average values of assimilating pigment quantities determined in extracts prepared from stem of *E. canariensis,* at 18 months of culture on MS-G medium prepared with distilled water (DW) (V_0 - control) or MS-G medium prepared with deuterium depleted water (DDW) (V_1).

Experimental type / Assimilating pigments	MS-G with DW (*control*)	MS-G with DDW	
	average (µg/g)	*average (µg/g)*	*% toward control*
Chl *a*	1.49±0.01	1.70±0.02 ns	114.1 %
Chl *b*	1.86±0.04	2.08±0.03 ns	111.8 %
Total Chl	3.35	3.78	112.8 %
Chl *a/b*	0,801	0,817	102,03%
Carotenoids	0.56±0.06	0.98±0.08*	174.6 %
Total assimilating pigments	3.91	4.76	121.7 %

Note: ns – no significant $p>0.05$; * = $p < 0.05$.

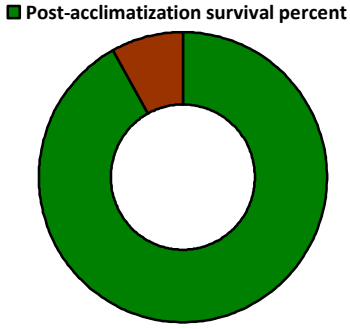

Figure 4. Post-acclimatization survival percent of *E. canariensis* plantlets from *in vitro* medium prepared with deuterium depleted water (V_1).

Thus, we have demonstrated that the initial hypothesis is true. In previous experiments we have increased the *ex vitro* survival rate of *Chrysanthemum* and *Saintpaulia ionantha* by watering their aerial parts, after transferring into soil, with DDW (Petrus - Vancea *et al.,* 2003; Petruş –Vancea *et al.,* 2013).

The results were positive in terms of the post-acclimatization survival rate, growth and ability to adapt to environmental conditions. All these results, obtained over the years, are in consensus with our presented in this study.

Conclusions

Deuterium depleted water (with 25 ppm D) stimulated the *in vitro* rhizogenesis of *E. canariensis*, because it eliminated callus formation at the basal level of plantlets and increase in stems the quantity of assimilating pigments, especially of carotenoids.

E. canariensis plantlets cultivated *in vitro* on medium prepared with deuterium depleted water (with 25 ppm D) had a good *ex vitro* survival capacity.

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